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Enzymatic Studies on Natural Rubber Degradation by *Streptomyces* sp.

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ABSTRACT
Rubber products are widely used in our daily life these products are mainly made up of Natural rubber (NR) which is obtained from the latex of tree *Hevea brasiliensis* commonly called Rubber tree. The different rubber products are manufactured by using vulcanized natural rubber. After usage of these natural rubber products the disposal of these products is the worldwide solid waste problem. Microbial degradation is one of the solution to this problem which is mainly carried out by various microorganisms such as fungi and actinomycetes. During the present study an attempt was made to isolate rubber degrading microorganism. Rubber discs were dumped in the soil for regular interval of time and then plated on the media to isolate the organism. In the isolated organism *Streptomyces* sp. effectively degraded the natural rubber sample. The present study has showed that, it is possible to use this strain to degrade the natural rubber.
Key words: *Streptomyces* sp., *Hevea brasiliensis*, vulcanization, polyisoprene, Laccase, Manganese peroxidase.

INTRODUCTION

Rubber products are widely used in our daily life these products are mainly made up of Natural rubber (NR) which is obtained from the latex of tree *Hevea brasiliensis* commonly called Rubber tree. The average composition of the natural rubber latex is 25-30% polyisoprene, 1-1.8% proteins, 1-2% carbohydrates, 0.4-1.1% neutral lipids, 0.5-0.6% polar lipids, 0.4-0.6 inorganic components, 0.4% amino acids etc., and other 50-70% water. The natural rubber latex is sticky and viscous in nature and very sensitive to temperature therefore it can not be directly used for the manufacturing of rubber products. For the manufacture of rubber products this latex should be subjected to vulcanization.

The global rubber consumption is estimated to be 12.5 million metric tons in 2013 of which 65% were used for tire production and other 35% is used for the production of other rubber products. After usage of these natural rubber products, disposal of these products are the world wide solid waste problem. One of the solution to reduce this problem is to recycle the used waste rubber. But due to the chemical cross linking formed during vulcanization it is not possible to simply melt and reshape the products as in case of polythene. So other alternatives such as microbial degradation of the product should be developed. Microbial degradation is mainly carried out by various microorganisms such as fungi and actinomycetes.

The present study was taken to isolate the natural rubber degrading actinomycetes from the soil so that it can be used to degrade the rubber waste. In the present study an attempt was also made to study about the enzymes produced by *Streptomyces* sp. to degrade natural rubber.

MATERIALS AND METHODS

For the isolation of actinomycetes which were able to degrade natural rubber, the soil sample was collected from a local land fill of Shivamogga district and brought to the laboratory, preserved under laboratory conditions for further use. Along with this natural rubber latex and natural rubber sheet samples were collected from rubber processing unit and then it was brought to the laboratory and preserved in the refrigerator for further use.

Isolation of natural rubber degrading Actinomycetes

For the isolation of natural rubber degrading Actinomycete soil burial method was followed. Natural rubber small discs was weighed and initial weight was recorded. Then, these discs were dumped in the soil and left for a period of six months of time interval. These natural rubber discs were removed regularly at time interval of two, four and six months respectively and weighed. For the isolation of natural rubber degrading actinomycetes soil sample and natural rubber samples were plated on the starch casein agar and kept for incubation at room temperature or at 27±2ºC for 3 to 4 days for the isolation of actinomycetes [1]. After incubation period actinomycetes were identified by staining and based on their microscopic and macroscopic appearance using standard manuals [2].

Plate assay for the screening of Actinomycetes capable of degrading natural rubber

For the screening of natural rubber degrading actinomycetes pure culture isolates were directly inoculated on the sterilized, pre weighed natural rubber discs and then kept for incubation for 2 months. After a time interval of 2 months natural rubber sample inoculated with organisms were washed thoroughly, dried at 50°C in hot air oven for 24 hours and final weight was recorded [3].

Screening of natural rubber degradation by using Mineral salt medium (MSM)

Natural rubber degrading ability of the actinomycetes was checked in the laboratory conditions by growth experiment in mineral salt medium (MSM) [4], where natural rubber was used as sole carbon source. Previously isolated actinomycetes were inoculated to different conical flasks containing MSM and kept for incubation for 2 months on rotary shaker. Actinomycetes were incubated at 27±2°C, triplicates were maintained. After incubation period natural rubber discs were removed and observed for the growth of Actinomycetes. Then natural rubber discs were washed, dried at 50°C in hot air oven for 24 hours and weight loss was checked [5].

Confirmation of natural rubber degradation by staining with Schiff's reagent

Evidence for degradation and mineralization of *cis*-1,4-polyisoprene rubber hydrocarbon chain was obtained by staining treated natural rubber discs, which is containing actively growing colonies of microorganisms with Schiff's

reagent. In a tightly stopper bottle, 10 ml of fuchsin reagent was added to a sample and kept for incubation for 10-30 minutes at room temperature. After 10-30 minutes excess amount of the reagent was discarded and 10ml of the sulfite solution was added in order to suppress nonspecific reaction of untreated sample [6].

Confirmation of natural rubber degradation by Scanning Electron Microscopy (SEM)

Evidence for degradation and mineralization of *cis*-1,4-polyisoprene natural rubber hydrocarbon chain was obtained by observing the natural rubber discs under SEM. For the observation natural rubber discs buried in the soil and present in the MSM, which were subjected for degradation were observed under field emission-scanning electron microscopy (FEI-SIRION, Eindhoven, Netherland) [7].

Confirmation of natural rubber degradation by Fourier transform infrared spectroscopy (FTIR)

Chemical changes that arose directly on the natural rubber surface as result of the degradation process were determined using FTIR spectroscopy. NICOLET 380 FTIR spectrophotometer from Thermo Fisher Scientific, France was used which gives transmittance spectra in IR range 4000 to 400 nm. [8].

Confirmation of natural rubber degradation by Nuclear Magnetic Resonance spectroscopy (NMR)

Structural changes that arose directly on the natural rubber surface as result of the degradation process were determined using NMR spectroscopy. 400 MHz Supercon from Bruker, West Germany with multi nuclear probe commonly studies H1 NMR and ^{13}C NMR was used to study structural changes of rubber sample treated with microorganisms [9].

Characterization of enzymes responsible for biodegradation of natural rubber

It was studied that laccase and manganese peroxidase enzymes were responsible for the natural rubber degradation.

Screening for Laccase and Manganese peroxidase enzyme production by *Streptomyces* sp.

Screening for laccase enzyme produced by *Streptomyces* sp**.** was done on plates containing following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 Agar (pH-6) supplemented with 0.02% guaiacol. *Streptomyces* sp**.** was inoculated into this plate and the plate was incubated at 30°C for 7 days. Laccase activity was visualized on plates containing 0.02% guaiacol, since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium [10].

For the screening of manganese peroxidase enzyme producing organisms H_2O_2 was added to the laccase screening media.

Mass production of enzyme by submerged fermentation

Pure cultures *Streptomyces* sp**.** was inoculated to submerged state fermentation medium for the production of extracellular enzymes by using MSM media and was maintained at the incubation temperature of 27±2ºC for 3 months [11].

Determination of Laccase and Manganese peroxidase enzyme activity by using Spectrophotometer

Guaiacol (2mM) in sodium acetate buffer (10mM pH 5.0) was used as substrate. The reaction mixture contained 3ml 10mM acetate buffer of pH 5, 1ml guaiacol and 1ml enzyme source and enzyme blank contained 1ml of distilled water instead of enzyme source. The mixture was incubated at 30ºC for 15minutes. and absorbance was read at 450nm blank using UV spectrophotometer [12]. Manganese peroxidase enzyme activity was calculated by following laccase enzyme activity determination procedure, but for the reaction mixture 1 ml of H_2O_2 was added and incubated.

Protein Estimation

Protein concentration was estimated to determine specific activity of enzyme. The protein concentration was determined by the Lowry's method [13] using Bovine Serum Albumin (BSA) as a standard, absorbance was read at 660 nm using JENWAY- 6305 UV-VIS Spectrophotometer.

RESULTS

Rubber samples and the soil sample of 2, 4 and 6 months were plated on the starch casein agar, different actinomycetes were isolated and recorded. In the isolated organism *Streptomyces* sp., was pre-dominant and commonly isolated. Thus it was screened to test natural rubber degrading ability. Weight loss was also observed in all the rubber samples which was removed at different time interval (Table 1)

Plate assay for the screening of Actinomycetes capable of degrading natural rubber

In plate assay weight loss was observed in *Streptomyces* sp., inoculated natural rubber discs, initial weight of the rubber disc was 10g and final weight was 7.39g and there was decrease in 2.61±0.002 g weight and there was weight loss of 26.1%.

Screening of natural rubber degradation by using Mineral salt medium (MSM)

Growth experiment was conducted by using mineral salt medium weight loss was observed and growth of actinomycetes was observed on the natural rubber discs. Initial weight of *Streptomyces* sp., inoculated sample was 3g and final weight was 1.42g and there was a weight loss of 1.58g and there was a 52.6% weight loss.

Confirmation of rubber degradation by staining with Schiff's reagent

Natural rubber discs which were inoculated with microorganisms turned to purple colour and there was no colour formation in the untreated control. Formation of purple colour in the treated sample was due to the presence of aldehyde and ketone group, which was produced as a result of degradation of cis-1,4-polyisoprene units of natural rubber.

Confirmation of natural rubber degradation by Scanning Electron Microscopy (SEM)

Natural rubber discs were observed under SEM, bio-film formation, complete disintegration and formation of cavities on the natural rubber discs was observed (Fig. 1).

Confirmation of rubber degradation by Fourier Transform Infrared Spectroscopy (FTIR)

Natural rubber discs, which were treated by actinomycetes were subjected for FTIR studies peaks were observed at the wave length between 2723.29 cm^{-1} and 1660.27 cm^{-1} having H–C=O:C–H stretch and C=O stretch which indicates the presence of aldehydes and ketones, released as a result of natural rubber degradation in the treated sample. Presence of these aldehyde and ketone group confirms natural rubber degradation. Peaks showing the presence of aldehyde and ketone are absent in control (Fig. 2).

Confirmation of natural rubber degradation by Nuclear Magnetic Resonance spectroscopy (NMR)

Structural changes that arose directly on the natural rubber surface as result of the degradation process were determined using NMR spectroscopy. When rubber discs treated by microorganisms were subjected for NMR peaks showing the change in the structure of the rubber was observed. Presence of aromatic protons, NH protons, NH4 protons and aliphatic chains shows the degradation of natural rubber by microorganisms which was absent in control.

Enzymatic Studies of natural rubber degradation

It was studied that laccase and manganese peroxidase enzymes were responsible for the rubber degradation.

Screening for Laccase and Manganese peroxidase enzyme production by *Streptomyces* sp.

Streptomyces sp. was inoculated on the laccase and manganese peroxidase medium, there was a formation of reddish brown colour around the colonies, as laccase and manganese peroxidase catalyzes the oxidative polymerization of guaiacol to form reddish brown zone. *Streptomyces* sp. which showed positive result for rubber degradation showed positive result for laccase and manganese peroxidase enzyme screening.

Spectrophotometrical analysis of Laccase and Manganese peroxidase enzyme activity

Streptomyces sp., showed more manganese peroxidase activity compared to laccase activity. Maximum activity of both laccase and manganese peroxidase enzyme activity was maximum in 10^{th} week. Laccase enzyme activity in 10^{th} week was 0.0181 IU and manganese peroxidase activity in 10^{th} week was 0.0189 IU (Table 3).

Protein Estimation

Specific activity of laccase enzyme in *Streptomyces* sp. was 0.0150± 0.006µmol/ml/min/mg and specific activity of manganese peroxidase enzyme in *Streptomyces* sp. was 0.171±0.006 µmol/ml/min/mg**.**

DISCUSSION

Present study was carried out to isolate natural rubber degrading actinomycetes. It was studied that *Streptomyces* sp. is capable of degrading natural rubber. Degradation of natural rubber was studied by carrying out growth experiment in MSM, and degradation was confirmed by staining, SEM, FTIR and NMR studies. Further enzyme responsible for degradation was studied. Laccase and manganese peroxidase were the enzymes responsible for degradation.

Similar attempts were made by several other scientists to degrade rubber by using microorganisms.

An attempt to study on natural rubber (NR) biodegradation through solid-state fermentation (SSF) and submerged fermentation (SMF) has been carried out for both bacterial as well as fungal species. There was a change in the organic carbon content along with the average molecular weight of the treated rubber samples indicated rubber hydrocarbon utilization and its degradation [8].

Similar work was conducted to test the biodegrading ability of different actinomycetes belonging to the genera *Gordonia* (strains Kb2, Kd2 and VH2), *Mycobacterium*, *Micromonospora* and *Pseudomonas*. All strains were able to use natural rubber (NR) as well as NR latex gloves as sole carbon source [6].

Forty-seven percent of a tire tread strip with a natural rubber content of 100 phr (parts per hundred of rubber) was completely mineralized by a mutant strain, Rc, of the rubber-degrading organism, *Nocardia* sp. Strain 835A [14].

CONCLUSION

Rubber products are widely used in our daily life. These products are made up of natural vulcanized rubber and other chemical additives. Due to vulcanization of the natural rubber these rubber are very resistant to high temperature and persist in environment for very long time. Rubber materials have been increasingly used now a days in different area after usage its disposal is a very big solid waste problem. It cannot be easily recycled due to the sulphur cross linking formed during vulcanization. If they are burnt they release enormous amount of carbon-di-oxide and some other gases which causes environmental pollution and contribute to the global warming. Rubber products such as balloon which are disposed in the natural environment are considered to be dangerous to wild animals if they are consumed by animals.

So one of the alternative way to solve these problems is to subject these product to biodegradation. During the present study rubber discs were dumped in the soil were removed at regular interval of time and then plated on the media to isolate the organism. In the isolated organism and *Streptomyces* sp. effectively degraded the rubber sample. The present study has showed that, it is possible to use *Streptomyces* sp. to degrade natural rubber effectively. Along with this enzymes responsible for natural rubber degradation was also characterized.

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REFERENCES

[1] Tsuchii, A., Takeda, K., Suzuki, T. and Tokiwa, Y. 1996. Colonization and degradation of rubber pieces by *Nocardia* species, *Biodegradation Journal*, 7: 41-48.

[2] Peter, H.A., James, T. and Stanley, T. 1994. Bergey's manual of determinative bacteriology. 9th edition: 1-754.

[3] Borel, M., Kergomard, A., and Renard, F. 1981. Degradation of Natural rubber by actinomycetes imperfecti, *Agricultural Biology and Chemistry*, 46: 877-881.

[4] Pan, L., Gung, G.J., Yin, B. and Cheng, P.S. 2009. Contribution to deterioration of polymeric materials by slow growing Actinomycetes*Nocardia corynebacterioides*, *International Biodegradation and Biodeterioration*, 63: 24-29

[5] Tsuchii, A. and Tokiwa, Y. 2001. Microbial degradation of Tire rubber particles, *Biotechnology Letters*, 23: 963-969.

[6] Berekaa, M.M., Lions, A., Reichelt, R., Keller, U. and Steinbuchel, A. 2000. Effect of pretreatment of rubber material on its Biodegradability by various rubber degrading Bacteria, *FEMS Microbiology Letters*, 184: 199-206. .

[7] Lions, A., Rudolf Reichelt, R., Keller, U. and Steinbuchel, A . 2000. A Gram negative bacterium identified as *Pseudomonas aeruginosa*, AL98, is a potent degrader of natural rubber and synthetic cis-1,4-polyisoprene, *FEMS Microbiology Letters*, 182: 155-161.

[8] Roy, V.R., Das, M., Banrjee, R. and Bhowmick, A. 2005. Comparative studies on rubber biodegradation through Solid state and Submerged fermentation, *Process Biochemistry*, 42: 181-186.

[9] Shah, A.A., Hasan, F., Hameed, A and Ahmed, S. 2008. Biological degradation of Plastics: A comprehensive review. *Biotechnology Advances*, 26:246 265.

[10] Viswanath, B., Chandra, M. S., Pallavi, H., Reddy, 2008. Screening and Assessment of Laccase Producing Actinomycetes isolated from different environmental samples, *African Journal of Biotechnology*, 7:1129-1133.

[11] Shraddha, Shekher, R., Sehgal, S., Kamtania, M., and Kumar, A., 2011. Laccase microbial source Production, Purification, Potential biotechnological application, *Enzyme Research*, 1-11.

[12] Papinutti, L., and Martinez, J. M., 2006. Production and Characterization of Laccase and Manganese peroxidase from the ligninolytic fungus *Fomes sclerodermeus*, *Journal of technology and biotechnology*, 81:1064-1070.

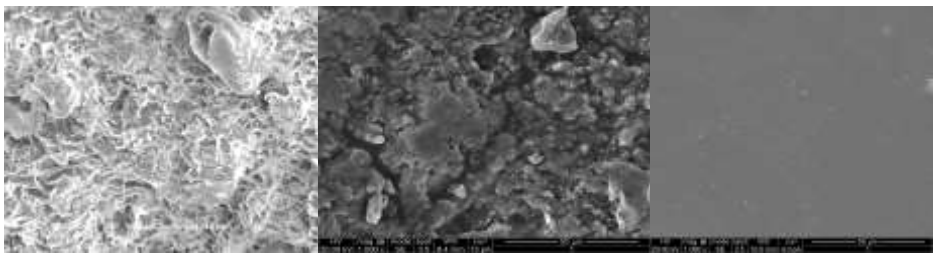
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., 1951. Protein measurement with the folin phenol reagent, *Journal of general Microbiology*, 31:3017-3027.

[14] Tsuchii, A. and Tokiwa, Y. 1999. Colonization and disinteragration of Tire Rubber by a colonial Mutant of *Nocardia*, *Journal of Bioscience and Bioengineering*, 87: 542-544.

Table 1. Weight loss of natural rubber by soil burial method

Sl. No.	Number of months	Initial weight (g)	Final Weight (g)	Weight loss (g)	Weight loss In (%)
1.	**2**	**3**	**2.84**	**0.16±0.01**	**5.3**
2.	**4**	**3**	**2.63**	**0.37±0.04**	**12.3**
3.	**6**	**3**	**2.12**	**0.88±0.01**	**29.3**

Result are expressed in standard error where n=3.



Buried in soil *Streptomyces* sp. treated Control

Fig. 1. SEM images of natural rubber showing degradation

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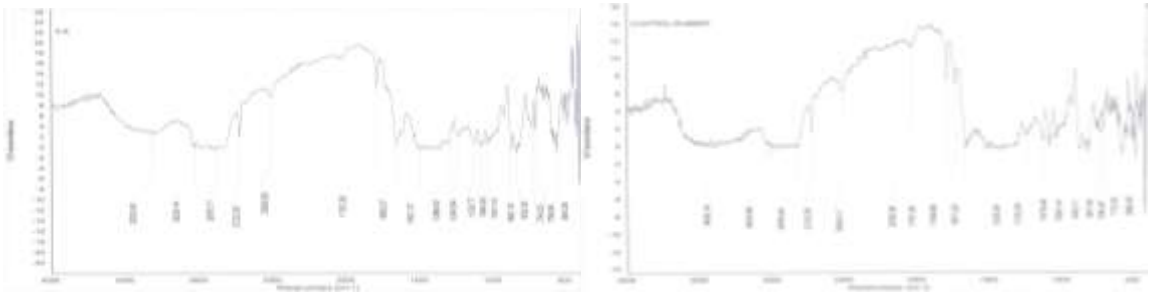

Streptomyces sp. treated Control

Fig. 2. Confirmation of natural rubber degradatiob by FTIR.

Control *Streptomyces* sp. treated

Fig. 3. Confirmation of natural rubber degradation by NMR.

Table 3. Laccase and Manganese peroxidase enzyme activity in IU.

Streptomyces sp.	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week	9th week	10th week	11th week	12th week
Laccase	0	0	0.0024	0.0035	0.0053	0.0083	0.0116	0.0146	0.0164	0.0181	0.0133	0.0111
Manganese peroxidase	0	0	0.0024	0.0038	0.0064	0.0093	0.0128	0.0147	0.0168	0.0189	0.0133	0.0111

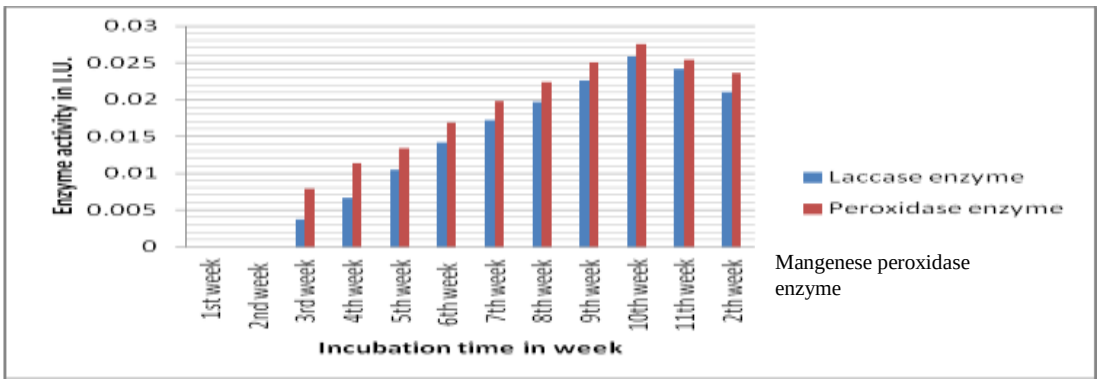

Fig. 4. Laccase and manganese peroxidase enzyme activity in IU.